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Adenosine A_1 receptors selectively target protein kinase C isoforms to the caveolin-rich plasma membrane in cardiac myocytes

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ABSTRACT

Adenosine is a naturally occurring nucleoside that has been shown to regulate a variety of functions in the cardiovascular system. However, the mechanisms in adenosine receptor signaling are not completely understood. Given that adenosine receptors have been linked to protein kinase C (PKC) in cardioprotection and caveolae is critical for receptor signaling, we sought to determine whether activation of adenosine A1 receptors induces selective translocation of PKC isoforms to the membrane from the cytosol and whether activated PKC is targeted to the caveolin-rich plasma membrane microdomains. The freshly isolated adult rat cardiac myocytes were used to examine PKC isoforms including PKCα, PKCβ, PKCε, PKCδ and PKCζ. Immunoblot analysis revealed that the immunoreactivity for PKCε or PKCδ but not for PKCα, PKCβ or PKCζ increased significantly in the membrane fractions from cells pretreated with the selective adenosine A1 receptor agonist 2-chloro-N(6)-cyclopentyladenosine (CCPA, 100 nM) when compared with non-stimulated cells. The effect of CCPA on PKCε or PKCδ translocation was blocked by adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 100 nM). When Western blot was performed from the caveolin-enriched plasma membrane fractions, the immunoreactivity for PKCε or PKCδ but not PKCα, PKCβ or PKCζ was enhanced significantly by CCPA. Furthermore, PKCε and PKCδ were detected in the anti-caveolin-3 immunoprecipitates but not in the samples without primary antibody. Immunofluorescence staining further indicates increased colocalization of PKCε or PKCδ with caveolin-3 at cell peripheral region and T-tubular-like structures in response to adenosine A1 receptor activation. In conclusion, we demonstrate that activation of adenosine A1 receptors promotes the selective translocation of PKCε and PKCδ to the caveolin-enriched plasma membrane microdomains in cardiac myocytes.

1. Introduction

Adenosine is a natural nucleoside produced from the cardiovascular system and exerts a variety of functions including bradycardia, hypotension and coronary vasodilation [1]. Adenosine recognizes specific cell surface receptors including four adenosine receptor subtypes (A1, A2A, A2B and A3). All these receptor subtypes are coupled to guanine nucleotide binding proteins (G proteins). The most characterized mechanism is the effect on adenylate cyclase. In addition, another important pathway involving protein kinase C (PKC) has been postulated to play a key role in adenosine receptor signaling [2–4].

PKC exists as a family of at least 12 isoforms. PKC isoforms mediate distinct cellular functions by phosphorylating specific downstream target proteins. Substrate specificity is most likely associated with subcellular localization of activated PKC isozymes. In the heart, a variety of signaling molecules have been localized in caveolae [5,6]. Caveolae are small (50 to 100 nm) cholesterol and sphingolipid enriched "cave"-like invaginations of the surface membrane, very rich in many of the signaling molecules [7–11]. These microdomains may act to generate subcellular signaling compartments by recruiting interacting signaling molecules. Indeed, cardiac myocyte caveolae are the focal points for activated PKC isoforms and their downstream signaling molecules [10].

PKC can be activated by either exogenous PMA or endogenous receptor-mediated signaling. While PMA activates both classical and novel PKC isoforms, translocation of PKC by distinct receptor signaling is isoform selective. Adenosine A_1 receptor is well known to couple to inhibitory G proteins, and inhibitory G protein often couple to their effectors by PKC [2,12,13]. It has been shown that PKCε translocation to cardiac sarcolemma or T-tubular structures is involved in adenosine receptor-mediated responses [14–16]. Recent evidence suggests that adenosine A1 receptor activation can selectively modify phosphatase and mitogen-activated protein kinases (MAPK) activities in caveolin-rich cardiac membrane fractions [17]. However, it is not known whether adenosine receptor activation targets translocated PKC isoforms to the caveolin-rich plasma membrane microdomains. To determine whether activation of adenosine A1 receptors promotes translocation and caveolar targeting of specific PKC isoforms, we

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employed the freshly isolated adult cardiac myocytes and examined whether activation of adenosine A_1 receptors by CCPA induces translocation of PKC isoforms to the cell membrane and whether translocated PKC isoforms by CCPA are targeted to caveolin-rich plasma membrane microdomains. We focused on examining six major isoforms of PKC that are known to be expressed in adult rat cardiomyocytes [18,19]. Our results demonstrate that activation of adenosine A_1 receptors increases immunoreactive membrane PKCε and PKCδ but not PKCα, PKCβ and PKCζ in the caveolin-rich microdomains.

2. Materials and methods

2.1. Materials

Mouse IgG directed against caveolin-3 and rabbit IgG directed against PKCε, PKCβ1, PKCβ2 or PKCδ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit IgG directed against PKCα was purchased from Cell Signaling Technology (Danvers, MA). Rabbit IgG directed against PKCζ was purchased from Santa Cruz Biotechnology or Sigma (St. Louis, MO). The phorbol ester phorbal-12-myristate-13-acetate (PMA), its inactive congener 4α-PMA, adenosine A1 receptor agonist 2-chloro-N(6)-cyclopentyladenosine (CCPA) and adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were supplied from Sigma. PMA, 4α-PMA, CCPA and DPCPX were all used at a final concentration of 100 nM. DPCPX was applied 5 min before and during addition of CCPA to cell suspension. All drugs were dissolved in dimethyl sulfoxide, which did not exceed a final concentration of 0.1%.

2.2. Isolation of cardiomyocytes

Adult rat ventricular myocytes were isolated from adult Sprague–Dawley rats (250 to 300 g) by enzymatic dissociation [20,21]. In brief, hearts were excised and retrogradely perfused via the aorta with oxygenated (100% O2) Tyrode's solution containing (in mM) NaCl 126, KCl 5.4, CaCl2 1.0, MgCl2 1.0, NaH2PO4 0.33, HEPES 10 and glucose 10 at 37 °C. The perfusate was then changed to a Tyrode's solution that is nominally Ca2+ free but otherwise has the same composition. The hearts were perfused with the same solution containing collagenase for 20 min. Softened ventricular tissues were removed, cut into small pieces and mechanically dissociated by trituration. The digested cells were initially stored in a solution containing (in mM) KCl 20, KH2PO4 10, glucose 10, potassium glutamate 70, β-hydroxybutyric acid 10, taurine 10, mannitol 5 and EGTA 5, along with 1% albumin, and then transferred to normal Tyrode solution. Cells were incubated in Tyrode solution with or without PMA, 4α-PMA, CCPA or CCPA plus DPCPX for 5 min at 37 °C prior to homogenization for subsequent biochemical and immunofluorescence experiments.

2.3. Purification of caveolin-enriched membrane fractions

Caveolin-rich fractions from adult rat cardiomyocytes were prepared by using a previously described detergent-free method with some modification [22]. Briefly, freshly isolated cardiomyocytes were pretreated without or with PMA or CCPA for 5 min at 37 °C. Cells were then spin down and resuspended in 0.5 M sodium carbonate and homogenized. The homogenate was adjusted to 45% sucrose by addition of an equal volume of 90% sucrose in MBS (25 mM Mes, pH 6.5/0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient (in MBS containing 250 mM sodium carbonate) was formed above by overlaying with 4 ml of 35% sucrose (prepared in MBS with 250 mM sodium carbonate) and then 4 ml of 5% sucrose (again prepared in MBS with 250 mM sodium carbonate). Tubes were centrifuged at 39,000 rpm for 18–20 h in an SW41 rotor. Twelve 1-ml fractions were collected from the top to the bottom of the gradient for subsequent analysis by Western blot. Caveolin-rich fractions (fractions 4–6) which contain caveolin but exclude most other cellular proteins were centrifuged at 40,000×*g* for 2 h to pellet caveolae, which was then suspended in lysis buffer and sonicated. Samples were resolved by SDS–PAGE, transferred onto a nitrocellulose membrane and analyzed by probing with various antibodies.

2.4. Membrane fractionation and western blotting

Freshly isolated cardiac myocytes were incubated in the presence and absence of PMA, 4α-PMA, CCPA or CCPA plus DPCPX for 5 min prior to homogenization. Cell lysates were first centrifuged at 1000×*g* to get rid of unbroken cells and nucleus. Particulate and cytosolic fractions were prepared by centrifugation at 27,000×*g* for 1 h or 45,000×*g* for 30 min. Immunoblot analysis was carried out as described previously [23,24]. Briefly, the cytosolic and particulate fractions were denatured in a sample buffer. Equal amounts of proteins were loaded and electrophoresed on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. The transferred blots were blocked with 5% nonfat milk in Tris-buffered saline (TBS, 150 mM NaCl, 20 mM Tris–HCl, pH 7.4) and incubated for 2 h at room temperature or overnight at 4 °C with primary antibodies in TBS, 0.1% Tween 20. After washing, the blots were reacted with peroxidase-conjugated secondary antibodies for 45 min and developed using the ECL detection system.

2.5. Co-immunoprecipitation

Immunoprecipitation experiments were performed as reported previously [22,25]. Cells were pretreated with or without CCPA prior to homogenization. The cell lysate was incubated with or without antibody against caveolin-3 for 2 h at 4 °C. Antigen–antibody complexes were captured with r-protein-A agarose (4 °C, 30 min). Agarose beads were washed 4 times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by SDS–PAGE, transferred onto a nitrocellulose membrane and analyzed by probing with antibodies against PKCε, PKCδ or caveolin-3.

2.6. Immunofluorescence confocal microscopy

As described previously [22], after pretreated cells with or without CCPA, the cells were fixed with 4% formaldehyde in PBS for 30 min, blocked, permeabilized in 5% goat serum in PBS with 0.1% Triton X-100 (30 min) and labeled with primary antibody for 2 h. Cells were then washed three times and labeled with fluorescence-conjugated secondary antibody for 1 h. Immunofluorescence was visualized with confocal laser scanning microscopy. All images were analyzed using a background subtraction method offline.

3. Results

3.1. Effect of PMA on translocation of PKC isoforms from the cytosol to the particulate fraction

To investigate whether activation of adenosine receptors induces translocation of PKC isoforms from the cytosol to the particulate (membrane) fraction, we first examined the subcellular localization of a representative isoform from each PKC subgroup, i.e. PKC-α (the classic PKC), -ε (the novel PKC) and -ζ (the atypical PKC) following PMA (100 nM) treatment [26]. It is known that PMA activates the classical and novel but not the atypical PKC isoforms. As expected, Western blot analysis of cytosolic and membrane fractions separated by high speed centrifugation from rat cardiomyocytes detected all

three PKC isoforms: PKCα, PKCε and PKCζ. Immunoblots with antibody against PKCα revealed a prominent protein band, which migrated with an apparent molecular mass of ~80 kDa. Antibody directed against PKCε detected a 90-kDa band while PKCζ was detected as a protein with a molecular mass of ~78 kDa and appears as doublets. Our results revealed that PMA pretreatment (37 °C, 5 min) induced a significant redistribution of PKCα and PKCε from the cytosol to the membrane but not PMA-insensitive isoform PKCζ (Fig. 1A). The membrane to cytosol ratios of both PKCα and ε increased approximately threefold in PMA-treated cells when compared with control group (Fig. 1B). The inactive congener 4α-PMA did not cause translocation of these PKC isoforms. Thus, we are able to demonstrate that PMA induces translocation of PKCα and PKCε but not PKCζ under our experimental condition.

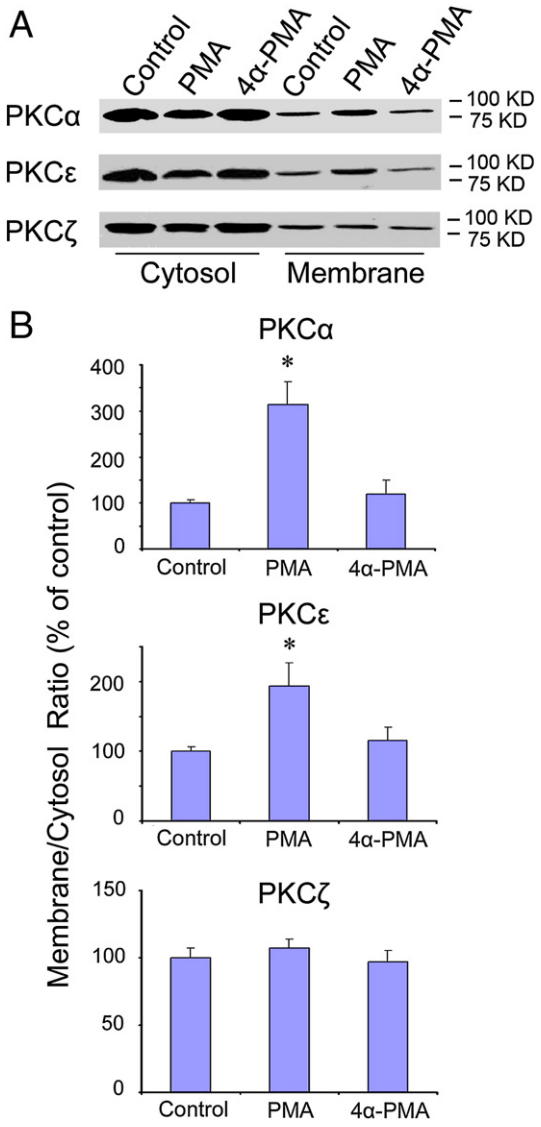

Fig. 1. Subcellular redistribution of PKC isoforms in response to PMA. (A) Representative Western blots of three to four independent experiments (three to four hearts). (B) Membrane to cytosol ratio as indexes of PKC isoform translocation. They were calculated by relative densitometry and normalized to 100% of control. Soluble and particulate fractions were prepared from cells incubated at 37 °C for 5 min in the absence or presence of PMA (100 nM) or 4α-PMA (100 nM). Equal amounts of total proteins were loaded in each lane, separated by SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were probed with antibodies directed against PKCα, PKCε or PKCζ and incubated with horseradish peroxidase (HRP)-linked secondary antibodies. Immunoblots were detected with enhanced chemiluminescence and multiple exposures to film. *$p<0.05$.

3.2. Effect of adenosine A1 receptors on translocation of PKC isoforms from the cytosol to the particulate fraction

We then determined the effect of adenosine A1 receptors on subcellular distribution of PKC isoforms. We examined six PKC isoforms that are present in the adult rat cardiomyocytes, including PKCα, PKCβ1, PKCβ2, PKCε, PKCδ and PKCζ. Cells were incubated with or without the selective A1 receptor agonist CCPA (100 nM) or CCPA plus the selective A1 receptor antagonist DPCPX (100 nM) for 5 min at 37 °C prior to homogenization and fractionation. The purity of the membrane or cytosolic fractions was examined with α-tubulin and Na/KATPase (Fig. 2A). Western blot from cell homogenates revealed significant translocation of PKCε and PKCδ from the cytosol to the membrane in CCPA-treated group (Fig. 2B). It was noted that the CCPA-induced translocation of PKCε or PKCδ was moderate when compared with PMA-treated group. Densitometric analysis indicated that the membrane to cytosol ratio was increased by 79% ($179.16 \pm 21.52\%$, $n=3$, $p<0.05$ vs. control) for PKCε and 81% ($181.26 \pm 14.82\%$, $n=3$, $p<0.05$ vs. control) for PKCδ in response to CCPA (Fig. 2C). When data were normalized to control within the cytosol or membrane fraction, the CCPA treatment significantly increased membrane-associated immunoreactivity by 49% ($149.43 \pm 10.78\%$, $n=3$, $p<0.05$ vs. control) for PKCε and 66% ($166.46 \pm 23.18\%$, $n=3$, $p<0.05$ vs. control) for PKCδ. The signal from the cytosolic fraction was decreased by 16% for PKCε ($84.12 \pm 7.43\%$, $n=3$, $p=$NS vs. control) and 10% for PKCδ ($90.10 \pm 1.40\%$, $n=3$, $p=$NS vs. control). Thus, CCPA produce a decreased but not statistically different signal in the cytosolic PKCε and PKCδ. Treatment with CCPA plus adenosine A1 receptor antagonist DPCPX (100 nM) significantly prevented CCPA-induced translocation of PKCε and PKCδ. The membrane to cytosol ratio for PKCε was $116.80 \pm 8.33\%$ ($n=3$, $p=$NS vs. control) and for PKCδ was $129.48 \pm 11.07\%$ ($n=3$, $p=$NS vs. control) in the presence of CCPA and DPCPX. Thus, our data show that CCPA promoted the selective translocation of PKCε and PKCδ, the effect was blocked by DPCPX. Further, the subcellular distribution of PKCα, PKCβ1 or PKCβ2 was not altered significantly by CCPA treatment, suggesting that these PKC isoforms may not be as important as PKCε and PKCδ in adenosine receptor signaling in adult rat cardiomyocytes. As expected, our data also showed that CCPA had no apparent effect on the redistribution of diacylglycerol-insensitive PKCζ. These results suggest that activation of adenosine A1 receptors promotes selective translocation of PKCε and PKCδ but not PKCα, PKCβ or PKCζ to the membrane from the cytosol in the adult rat cardiomyocytes.

3.3. Effect of adenosine A1 receptors on targeting PKC isoforms to the caveolin-rich plasma membrane

Translocation to different cellular compartment enables PKC to colocalize with both activators and substrate. Since adenosine A_1 receptors have been shown to reside in the caveolae of cardiac myocytes [8], we would predict that their downstream signaling molecules such as PKC isoforms would specifically target these microdomains of plasma membrane. We have previously shown that using a detergent-free sucrose gradient extraction procedure, the muscle-specific caveolin-3 isoform is enriched in fractions 4–6 from adult rat cardiomyocytes [22]. Thus, in the present study caveolin-rich fractions 4–6 were collected to determine alterations in PKC isoform expression from cardiomyocytes with and without PMA or CCPA stimulation. The blots were probed with anti-caveolin-3 antibody to confirm the caveolin-rich fractions. Freshly isolated adult cardiomyocytes were incubated with PMA (100 nM) or CCPA (100 nM) for 5 min at 37 °C prior to fractionation. As shown in Fig. 3A, the antibody against caveolin-3 detected abundant caveolin-3, a signature protein for the caveolae of

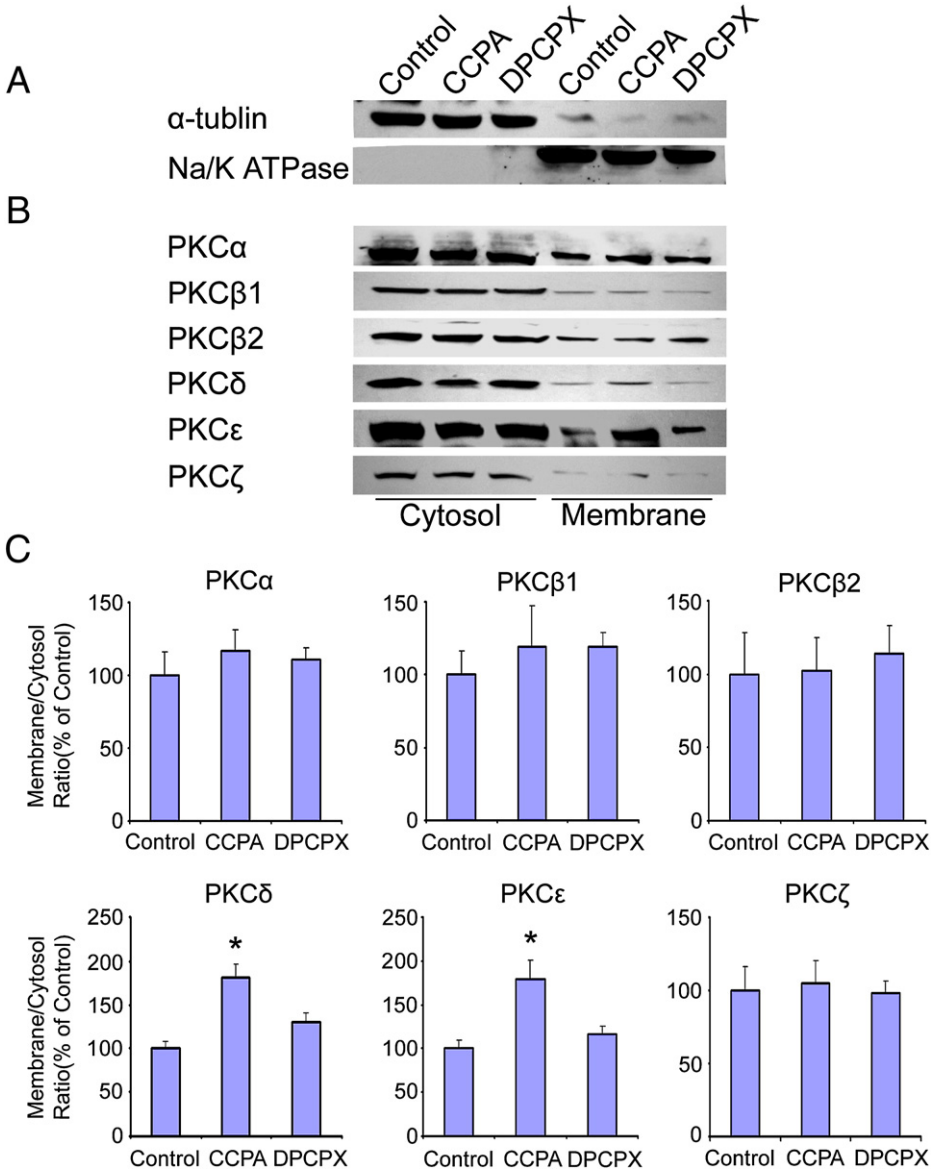

Fig. 2. Subcellular redistribution of PKC isoforms in response to CCPA. (A) Purity of the membrane or cytosol fraction. (B) Representative Western blots of three independent experiments (three hearts). (C) Membrane to cytosol ratio calculated by relative densitometry and normalized to 100% of control as indexes of PKC isoform translocation. Cells were incubated without or with CCPA (100 nM) or CCPA plus DPCPX (100 nM) for 5 min at 37 °C and then fractionated into soluble and particulate fractions. The adenosine A1 selective agonist CCPA induced significant translocation of PKCε and PKCδ (but not PKCα, PKCβ and PKCζ) from the cytosol to the membrane. The adenosine A_1 receptor antagonist DPCPX prevented CCPA-induced translocation of PKCε and PKCδ. Equal amounts of total proteins were loaded in each lane. *$p<0.05$.

cardiomyocytes, indicating that isolated fractions were caveolin-enrich fractions. Under control condition, only small amounts of immunoreactivity for all six PKC isoforms were detected in the caveolin-rich fractions. However, the band intensity for PKCα, PKCβ, PKCδ or PKCε increased significantly in response to the stimulation with PMA. Activation of adenosine A1 receptors with CCPA showed less but significant increase for PKCε and PKCδ. Densitometric analysis revealed that CCPA treatment enhanced the expression of PKCε in the caveolin-rich fraction by 105% ($205.59 \pm 51.79\%$, $n=3$, $p<0.05$ vs. control) and PKCδ by 53% (152.50 ± 16.86, $n=3$, $p<0.05$ vs. control), while the levels of immunoreactivity for PKCα, PKCβ1 or PKCβ2 were not altered significantly (Fig. 3B). As predicted, incubation with either PMA or CCPA did not lead to the recruitment of diacylglycerol/PMA-insensitive atypical PKCζ to the caveolin-rich domains. These results indicate that activation of adenosine A1 receptors promotes rapid association of PKCε and PKCδ with caveolin-rich plasma membrane microdomains.

3.4. Effect of adenosine A1 receptors on co-immunoprecipitation of PKCε and PKCδ with caveolin-3

PKC isoforms have been shown to localize to caveolae and interact with caveolin in a caveolin-subtype- and PKC isoform-dependent manner [27,28]. To further determine whether activated PKCε or PKCδ associates with caveolin-3 in cardiac myocytes and whether this association can be up-regulated by activation of adenosine A1 receptors, we performed co-immunoprecipitation in the cell lysates from cardiac myocytes pretreated with or without CCPA (100 nM). Cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-caveolin-3 antibody. The immune complex was collected with protein A agarose beads. Samples were resolved, transferred and analyzed by immunoblotting with antibody against PKCε, PKCδ or caveolin-3. As shown in Fig. 4, caveolin-3 co-precipitated with PKCε and PKCδ under basal condition. Both proteins were not detected in the lysates without immunoprecipitation with anti-caveolin-3 antibody. Interestingly, pretreatment with CCPA

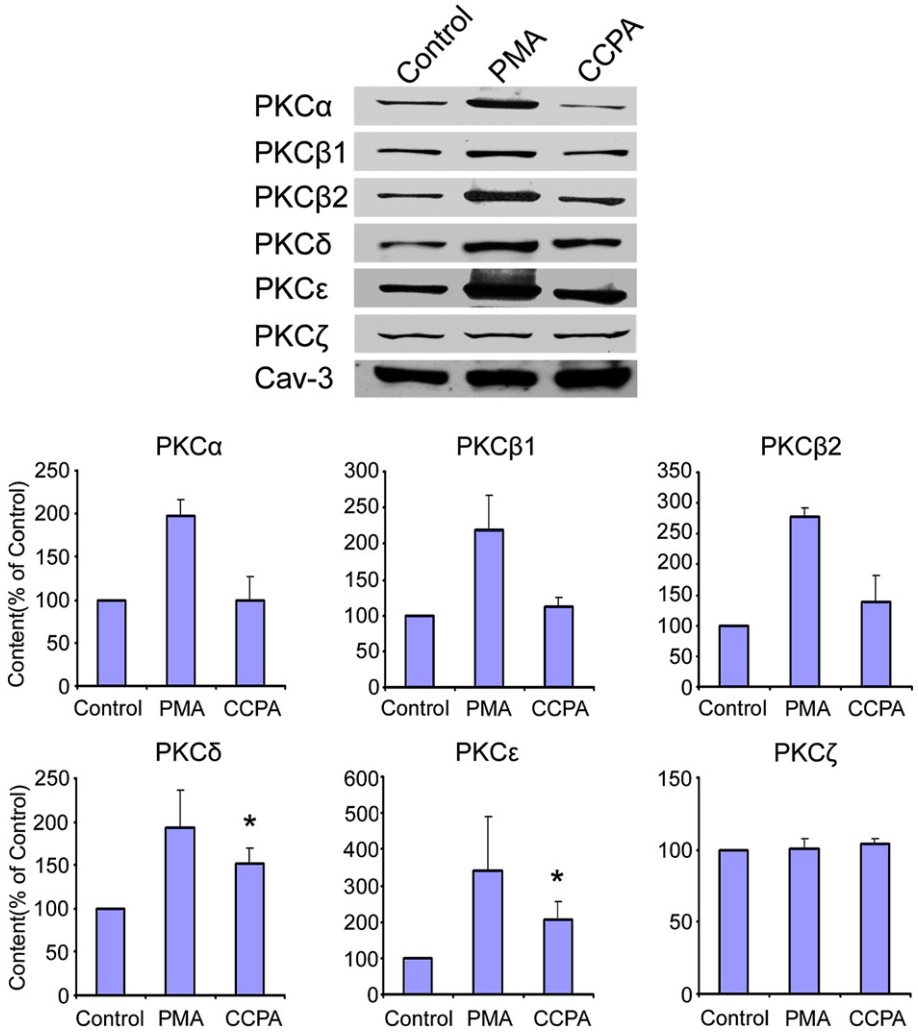

Fig. 3. PKC isoforms translocated to caveolin-rich microdomains in response to stimulation with CCPA. (A) Representative Western blots of three independent experiments from caveolin-enriched fractions. (B) Expression of PKC isoforms in caveolin-rich fractions calculated by relative densitometry and normalized to 100% of control. Cells were incubated in the absence or presence of PMA (100 nM) or CCPA (100 μM) at 37 °C for 5 min before processed for purification of caveolin-rich fractions. The caveolin-rich fractions 4–6 by sucrose gradient centrifugation were centrifuged at 40,000×*g* for 2 h to pellet caveolae, which was then suspended in lysis buffer and sonicated. Equal amounts of caveolae proteins were loaded and resolved by 10% SDS–PAGE, transferred onto nitrocellulose membrane and analyzed by probing with various antibodies. Similar to PMA, CCPA caused significant increase in the expression of PKCε and PKCδ in the caveolin-rich fractions although the degree of targeting PKC isoforms to caveolae by CCPA was less than that by PMA. Caveolin-rich fractions were verified by the heavy bands detected by anti-caveolin-3 antibody. *$p<0.05$.

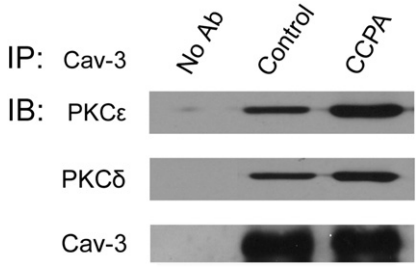

Fig. 4. Effect of CCPA on co-precipitation of caveolin-3 with PKCε or PKCδ. After pretreatment with or without CCPA (100 nM), cells were homogenized. Cell lysates containing equal amounts of total proteins were incubated with or without anti-caveolin-3 (Cav-3) antibody 2 h at 4 °C. Antigen–antibody complexes were captured with r-protein-A agarose for 30 min at 4 °C. Agarose beads were washed 4 times with solubilization buffer before removal of bound proteins by boiling in SDS sample buffer. Samples were resolved by 10% SDS–PAGE, transferred onto nitrocellulose membrane and analyzed by probing with antibody against PKCε, PKCδ or caveolin-3. A smaller amount of PKCε and PKCδ remained constitutively associated with caveolin-3 under the basal condition. CCPA increased the translocation and association of PKCε and PKCδ with caveolin-3.

significantly increased PKCε level by 67% ($167.31 \pm 8.65\%$, $n=7$, $p<0.05$ vs. control) and PKCδ by 53% ($153.67 \pm 7.34\%$, $n=6$, $p<0.05$ vs. control) in the caveolin-3 immunoprecipitates. CCPA did not alter the recovery of caveolin-3. These data demonstrate that activation of adenosine A1 receptors with CCPA promotes the translocation and association of PKCε and PKCδ with caveolin-3-rich membrane fraction in adult rat cardiomyocytes.

3.5. Effect of adenosine A1 receptors on colocalization of PKCε and PKCδ with caveolin-3

To further confirm PKCε and PKCδ localize to caveolin-rich microdomains, we assessed whether both isoforms colocalize with the marker protein of cardiomyocyte caveolae, caveolin-3, after stimulation of adenosine A1 receptors with CCPA at 100 nM. Fig. 5 shows confocal immunofluorescence images for PKCε, PKCδ and caveolin-3 from adult rat ventricular myocytes. These images revealed that the limited basal levels of PKCε and PKCδ were present in the caveolin-3-associated membrane in the cells without CCPA pretreatment, as indicated by a few yellow punctate staining along the cell peripherals. The staining pattern with anti-PKCε or anti-PKCδ

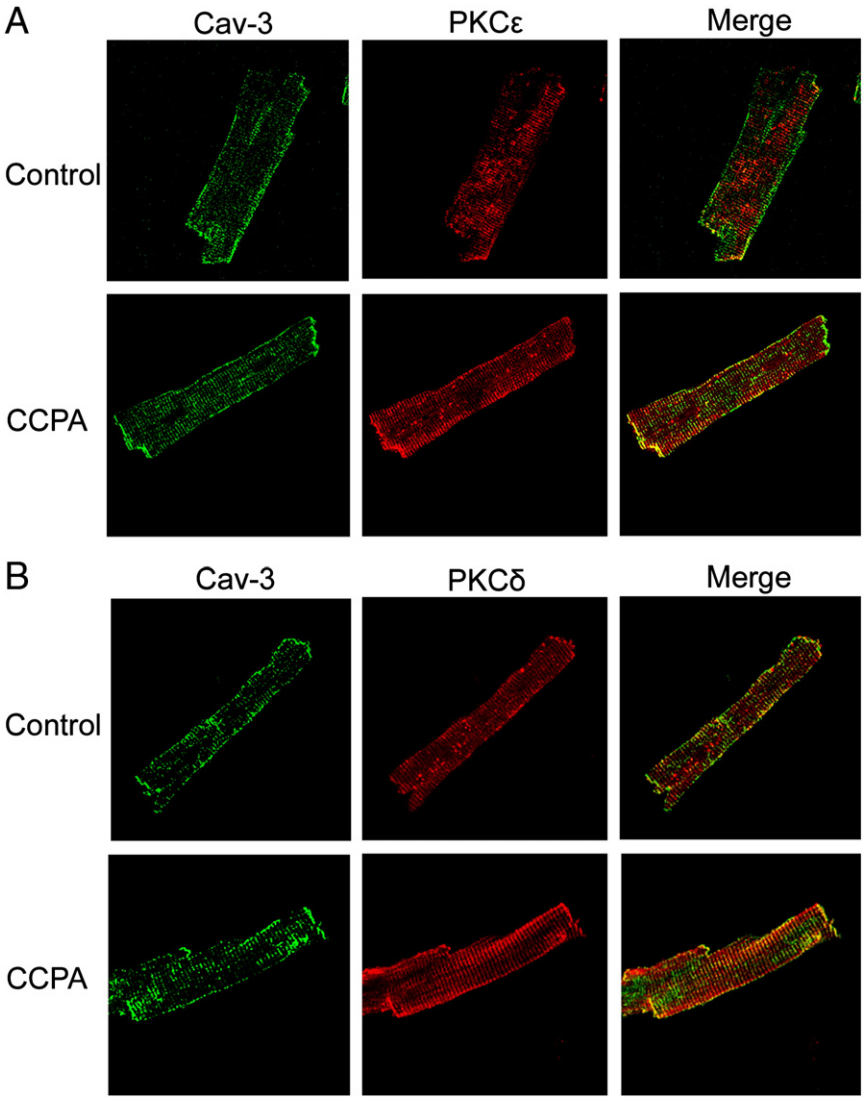

Fig. 5. Confocal laser microscopic imaging of adult rat cardiomyocytes in response to CCPA. Cells were incubated with or without CCPA (100 nM) for 5 min at 37 °C before they were subject to permeabilization and immunostaining with antibody against PKCε, PKCδ or caveolin-3 (Cav-3). Both PKCε and PKCδ was predominantly associated with cytosol in unstimulated cells but translocated to cell peripherals and T-tubular-like structures on stimulation with CCPA. Results are representative of three independent experiments.

antibody demonstrated that both PKCε and PKCδ are mostly diffused throughout of cells and also located at cross-striated structures. Following CCPA stimulation, the antibody against PKCε demonstrated a prominent surface sarcolemmal punctate staining area. The merged images showed significant areas of colocalization for PKCε and caveolin-3 along the cell peripherals, T-tubular-like structures and cell-to-cell contact. A similar staining pattern of PKCδ by CCPA was observed except that PKCδ has more pronounced T-tubular staining when compared with PKCε. A total of 40 cells was imaged and analyzed. While 25% of cells for PKCε and 30% for PKCδ showed significant colocalization in the control group, the number of cells showing colocalization with caveolin-3 in CCPA-treated groups was increased to 83% for PKCε and 73% for PKCδ. These results indicate that activation of adenosine A1 receptors induces recruitment of PKCε and PKCδ to the caveolin-3-associated plasma membrane domains.

4. Discussion

The present study demonstrated that activation of adenosine A1 receptors promotes targeting of novel PKC isoforms, PKCε and PKCδ to caveolin-rich plasma membrane microdomains. We demonstrated that activation of adenosine A1 receptors induced a rapid association of PKCε or PKCδ with caveolin-3 in adult rat cardiac myocytes. Specifically, we found that activation of adenosine A1 receptors with CCPA induced the selective translocation of PKCε and PKCδ (but not PKCα, PKCβ and PKCζ) from the cytosol to the membrane. Importantly, we showed that activated PKCε and PKCδ were targeted to the caveolin-rich plasma membrane microdomains. We have also showed that PKCε and PKCδ colocalize to and associate with caveolin-3. This observation is consistent with the notion that activated PKC isoforms are recruited to caveolae via signaling mechanisms that are distinct among different PKC isoforms.

It is known that membrane-bound translocation of PKC from cytosol is a hallmark of PKC activation [29]. Translocation to different cellular compartments enables PKC to colocalize with both activators and substrates. Individual PKC isoforms are believed to mediate distinct cell functions. Upon stimulation, they are directed to distinct subcellular membrane regions by binding to their specific activated C kinase. The activated isoforms are anchored close to their particular substrates. Although pharmacological evidence suggests that adenosine receptors are linked to PKC activation [2,4], limited study has directly addressed translocation of PKC isoforms by adenosine receptors. Importantly, no study has ever explored the caveolar targeting of specific PKC isoform(s) induced by adenosine receptors.

In the present study, we examined the effect of adenosine A1 receptors on the selective translocation and caveolar targeting of PKC isoforms. We found that under basal conditions, the level of immunoreactivity for PKCε and PKCδ in cardiomyocyte caveolae was limited but enhanced significantly following stimulation of adenosine A1 receptors with CCPA. Other PKC isoforms tested including PKCα, PKCβ and PKCζ were not significantly translocated to the cell membrane from the cytosol by stimulating adenosine A1 receptors. These observations provide the novel evidence that activation of adenosine A1 receptors induces the selective translocation of PKCε and PKCδ but not PKCα, PKCβ and PKCζ in adult rat cardiomyocytes and the translocated PKCε and PKCδ are targeted to the caveolin-rich plasma membrane. We chose a 5-min treatment with CCPA because our preliminary data showed that 5-min incubation with CCPA induced the maximum translocation of PKC isoforms.

Although our data demonstrate that activation of adenosine A1 receptors promotes translocation of PKCε and PKCδ but not PKCα, PKCβ and PKCζ, these results do not exclude the possibility that other adenosine receptors may cause translocation of one or more of these PKC isoforms. For example, A3 receptors are linked to activation of PKCδ [30]. It is likely that the same PKC isoforms activated by different adenosine receptors may associate with distinct downstream signaling pathways and serve distinct roles. It is also possible that activation of the same adenosine receptors stimulates more than one PKC isoforms, each serving a different role under certain physiological conditions [31,32]. In addition to translocation of PKC isoforms, our finding indicates the importance of caveolae microdomains in the adenosine receptor-mediated translocation of PKC. A1 receptor activation has been shown to increase PKCδ in the membrane fraction [3] and PKCε in the membrane and T-tubules of cardiac myocytes [14,15]. These observations are consistent with our finding that activation of adenosine A1 receptors promotes translocation of PKCδ or PKCε to the caveolin-rich membrane fractions given that caveolae are also localized to the T-tubules [33]. However, Lester and Hofmann report that adenosine receptor activation causes translocation of PKCε but not PKCδ [14]. The discrepancy between this study and the present study may be due to different adenosine receptor agonist used. We used CCPA (100 nM) which is a selective adenosine A1 receptor agonist while Lester et al. employed N6-(2-phenylisopropyl)-adenosine R-(−)isomer (R-PIA) at 100 μM that could activate both A1 and A3 receptors as authors claimed. Even though different PKC isoforms may be targeted to caveolae upon activation, activated PKC isoforms within caveolae may associate with distinct cellular functions due to differential targeting of separate populations of caveolae which may contain different sets of signaling proteins. PKCε and PKCδ are the major Ca^{2+}-independent PKC isoforms and the most abundant PKC isoform found in the adult rat cardiomyocytes. The adenosine receptor-induced caveolar targeting of PKCε and PKCδ may have important implications in adenosine-mediated cardioprotection [18].

It is known that upon activation, PKC isoforms translocate to multiple subcellular sites including plasma membrane, mitochondria or nucleus. Differential targeting of activated PKC isoforms to distinct subcellular localization determines their substrate specificity. In the present study, we focused on examining PKC translocation to the caveolar plasma membrane since the intact caveolae structure in cardiac myocytes has been shown to be required for the cardioprotection of ischemic preconditioning [34,35]. Our observation that adenosine A1 receptors promote the selective translocation of PKCε and PKCδ to caveolar plasma membrane microdomain implies that caveolae may serve as a focal point for efficient signaling transduction involving adenosine A1 receptors. Further, both PKCε and PKCδ have generated considerable interest in cardiac signaling because of their association with ischemic preconditioning [36]. Of particular interest is the suggestion that a delay in the onset of cardioprotection is due to the time taken for PKC isoform translocation and that the memory of preconditioning is due to PKC dwelling in a translocated state [37]. Nevertheless, our results do not exclude the possibility that activation of adenosine A1 receptors may promote translocation of PKCε or/and PKCδ to other subcellular sites such as mitochondria or nucleus. In fact, mitochondrial translocation of PKCε has been linked to cardioprotection although molecular mechanism by which PKCε targets to mitochondria remains elusive [38,39].

In conclusion, our data demonstrate that activation of adenosine A1 receptor promotes selective translocation and caveolar targeting of PKCε and PKCδ but not PKCα, PKCβ and PKCζ. This finding provides new mechanistic insight into our understanding the role of caveolae in adenosine receptor-mediated PKC signaling and may have important implication in cardioprotection.

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